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The purpose of these studies was to determine the mechanisms governing the toxigenicity of food bacteria such as Clostridium botulinum and closely related organisms. Results from these studies show that C. botulinum types C and D cease to produce their dominant toxins when they are cured of their prophages. These nontoxigenic derivatives then become sensitive to bacteriophages of other cultures which induce the production of different toxins. One cured strain of type C was shown to be sensitive to bacteriophages from C. botulinum types C and D and C novyi type A. These bacteriophages induced the production of toxin of C. →

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20. ABSTRACT continued

botulinum type C or D or the alpha toxin of C. novyi, respectively. This same cured type C strain could simultaneously carry bacteriophages of type C and C. novyi type A or type D and C. novyi type A which simultaneously induced the production of both C. novyi alpha and botulinum toxins. When type C and D cultures were cured of their prophages, then minor toxins previously masked by the dominant toxins could be detected. As a result, types C and D can each be subdivided into eight subtypes based upon the different toxin combinations. Only two subtypes of type C and one subtype of type D were previously recognized.

C. botulinum type A and F strains have been cured of their prophages but they continue to produce toxin. Certain strains of nonproteolytic type B have ceased to produce toxin when they are cultured in medium containing acridine orange, but these strains are not sensitive to bacteriophages. Plasmids have been detected in these strains of types A, F, and B but the role these plasmids play is not known.

MECHANISMS OF TOXIN PRODUCTION OF FOOD BACTERIA (CLOSTRIDIUM BOTULINUM)

FINAL SUMMARY

DR. MELVIN W. EKLUND, FRANK POYSKY, MARK PETERSON

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NORTHWEST AND ALASKA FISHERIES CENTER
NATIONAL MARINE FISHERIES SERVICE
UTILIZATION RESEARCH DIVISION
2725 MONTLAKE BOULEVARD EAST
SEATTLE, WASHINGTON 98112

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INTRODUCTION

Clostridium botulinum is a pathogenic anaerobe that is characterized by its ability to produce powerful neurotoxins. Botulism is generally regarded as a foodborne intoxication resulting from the ingestion of a food containing a specific preformed toxin produced by one of the several known types of *C. botulinum*. Because of the lethal effect of the neurotoxin, growth and toxin production by *C. botulinum* in different food products have been of worldwide concern to food processors and consumers for many years.

In addition to foodborne botulism, wound and infant botulism occur in man. In wound and infant botulism, the neurotoxin is produced *in vivo* by the growth of *C. botulinum* in infected tissues and in the intestinal tract, respectively (1,8). Infant botulism was only recognized as a distinct clinical entity in 1976 (1) and has become the most important source of human botulism in the U.S.A.

Botulism also occurs in both domestic and wild animals in many different areas of the world. In cases of avian botulism, thousands of wild ducks and broiler chickens can be involved (2) resulting in serious financial losses and animal mortalities.

The quality and safety of food products are major factors in our environment that affect the health of our people. A natural phenomenon occurs which controls the ability of *C. botulinum* to produce toxins. In nature, *C. botulinum* frequently loses its ability to produce toxin as exemplified by the frequent occurrence of nontoxigenic organisms in certain locations. In laboratory culture, some strains also spontaneously lose their ability to produce toxin. There is therefore a need to understand the mechanisms that govern the toxigenicity of *C. botulinum* bearing in mind that an understanding of

these factors could give us an insight to procedures for controlling toxin production by C. botulinum. This understanding is of immediate importance to the food industry because the use of sodium nitrite as a food preservative has become a matter of considerable concern. Its presence in foods has been implicated in the formation of nitrosamine compounds (9), several of which have been shown to be potent carcinogens in animals (6). The status of the continued use of nitrite is therefore quite uncertain. The primary reason for retaining the use of nitrite has been its proven effectiveness as an inhibitor of C. botulinum. This knowledge about the mechanisms governing the toxigenicity of C. botulinum could also be valuable in developing methodology for differentiating toxic and nontoxic cultures and determining whether or not nontoxic cultures have the potential of producing toxin. This is especially true in cases of infant and wound botulism in which C. sporogenes is frequently found with the toxic C. botulinum cultures.

SUMMARY OF RESULTS

Clostridium botulinum is a heterogeneous group of strains that is divided into types A through G based upon the antigenic specificity of the neurotoxins that are produced. The strains of these types can be separated into the following four groups according to their deoxyribonucleic acid homologies and biochemical, physiological, and serological characteristics (4,5,7,10,11,12,13,14).

Group 1. Proteolytic types A, A_F, B, and F

Group 2. Nonproteolytic types B, E, and F

Group 3. Nonproteolytic types C and D

Group 4. Type G

This close relationship between members within a group suggests that the mechanisms governing toxigenicity of one type may apply to all types within

the group. Many different strains of each type within a group therefore have been studied to determine the mechanisms governing this toxigenicity and interrelationships of the different types.

Mechanisms governing toxigenicity of C. botulinum types C and D and closely related C. novyi

The ability to cure type C and D strains of their prophages which govern production of the dominant C_1 and D toxins has enabled us to study the other minor toxins produced by these cultures. It has also enabled us to determine the interrelationship of types C and D and other closely related clostridia. Our studies have shown that type C and D strains ceased to produce the dominant C_1 and D toxins when they were cured of their tox^+ bacteriophages. The majority of the type C and some type D strains, however, continued to produce C_2 toxin. This was the first time that type D strains were recognized as C_2 toxin producers.

The mechanism governing the production of the trypsin-activated toxin designated as C_2 is not known. Recently two derivatives of type C strain 164 were isolated that had ceased to produce C_1, D, and C_2 toxins. These nontoxigenic derivatives were re-infected with tox^+ phages and were induced to produce the C_1 and D toxin but not the C_2 toxin. This is the first isolation of a $toxC_2^-$ derivative from a $toxC_2^+$ culture.

When certain cultures of types C and D were cured of their prophages, they concomitantly ceased to produce the dominant C_1 and D toxins and became indistinguishable with respect to toxins produced. This suggested that types C and D may arise from the same bacterial strain. Subsequent studies, in fact, have shown that an interconversion of C. botulinum types C and D will occur if a bacteriophage from one is substituted for a bacteriophage of the

other type (3). Phages from type C will induce the production of the dominant C_1 and minor D toxins, and phages from type D will induce the production of the dominant D and minor C_1 toxins.

When C. botulinum type C strain 162 was cured of its prophage, it simultaneously ceased to produce C_1 and D toxins and became sensitive to phages from C. botulinum types C (phage $3C^{tox+}$) and D (phage $1D^{tox+}$) or to phages of C. novyi type A (phage $NA1^{tox+}$). When this culture (HS37) was infected with these phages, it produced dominant C_1, minor D; or dominant D, minor C_1; or alpha toxin of C. novyi, respectively. Neither strain 162 nor its derivatives produced the C_2 toxin, but they did produce the gamma toxin of C. novyi A. When strain HS37 was infected with phage $NA1^{tox+}$, it became immune to the infectio.. by the homologous phage, but continued to be sensitive to phages 3C and 1D (Table 1). Strain HS37 therefore could simultaneously carry phages

Table 1. Sensitivity of a derivative of Clostridium botulinum type C to different bacteriophages

Indicator organism	Sensitivity to phages		
	NA1	1D	3C
HS37	+	+	+
HS37 (NA1)	-	+	+
HS37 (1D)	+	-	-
HS 37 (3C)	+	-	-

NA1 and 1D or phages NA1 and 3C. By substitution of the phages, strain HS37 was induced to produce the following toxins (Table 2). (Toxins are listed according to titer produced; C_1 and D toxins are C. botulinum type C and D toxins and gamma and alpha toxins are C. novyi toxins.) (1) HS37 produced gamma toxins; (2) HS37(3C) produced C_1, D, and gamma toxins; (3) HS37(1D) produced D, C_1, and gamma toxins; (4) HS37(3C,NA1) produced C_1, alpha, gamma,

and D toxins; (5) HS37(1D,NA1) produced alpha, D, gamma, and C_1 toxins; and (6) HS37(NA1) produced alpha and gamma toxins. The toxigenicity and type of toxin produced by these cultures depended upon the continued presence of specific bacteriophages.

Table 2. Different toxins produced by a derivative of Clostridium botulinum type C infected with different phages

Culture	Lethal toxins produced[a]			
	C_1	D	Gamma	Alpha
HS37	-	-	+	-
HS37 (3C)	+[b]	+	+	-
HS37 (NA1)	-	-	+	+
HS37 (NA1,3C)	+[b]	+	+	+
HS37 (NA1,1D)	+	+[b]	+	+
HS37 (1D)	+	+[b]	+	-

[a] C_1 and D are C. botulinum types C and D toxins. Gamma and alpha are C. novyi type A toxins.
[b] Dominant toxin produced by the bacterial strain.

The titers of the toxins produced by strains HS37 infected with phages NA1 and 3C or NA1 and 1D are summarized in Tables 3 and 4. When HS37 was

Table 3. Toxins produced by strain HS37 (NA1,3C)

Alpha C. novyi toxin	Botulinum type C toxin
100 MLD/ml	20,000 MLD/ml

infected with phages NA1 and 3C, the C_1 toxin was dominant; but when HS37 was infected with NA1 and 1D phages, the alpha toxin of C. novyi was dominant.

When C. botulinum type C and D cultures are cured of their bacteriophages, they simultaneously cease to produce the dominant C_1 and D toxins. These

Table 4. Toxins produced by strain HS37 (NAl,1D)

Toxin treatment	Alpha C. novyi toxin	Botulinum type D toxin
Untrypsinized	2,000 MLD/ml	10 MLD/ml
Trypsinized	----------	500 MLD/ml

bacteriophage-sensitive strains continue to produce other minor toxins which were previously masked by the dominance of the C_1 and D toxins. These strains have also become sensitive to bacteriophages of other bacterial strains which induce the production of additional toxins. Based upon the production of different toxins and toxin combinations, 8 subtypes of C. botulinum type C and eight subtypes of type D are now recognizable (Tables 5 and 6). Types C and D share 3 of these subtypes. Prior to these studies, only type D and 2 subtypes of type C were recognized.

Table 5. Toxins produced by Clostridium botulinum type D and nontoxigenic strains infected with different bacteriophages

Culture	Lethal toxins produced[a]				
	D[b]	C_2	C_1	Gamma	Alpha
1873	+	+	+	-	-
Al13	-	+	-	-	-
South African	+	-	+	-	-
A020	-	-	-	-	-
HS37 (1D)	+	-	+	+	-
HS37[c]	-	-	-	+	-
HS37 (NAl,1D)	+	-	+	+	+
HS37 (NAl)[c]	-	-	-	+	+

[a] D, C_2, and C_1 are C. botulinum type C and D toxins. Alpha and gamma are C. novyi type A toxins.
[b] Dominant toxin produced by C. botulinum type D strains.
[c] Cured of phage 1D.

Table 6. Toxins produced by Clostridium botulinum type C and nontoxigenic derivatives infected with different bacteriophages

Culture	Lethal toxins produced[a]				
	C_1[b]	C_2	*D*	*Gamma*	Alpha
468C	+	+	+	-	-
AO28	-	+	-	-	-
162	+	-	+	+	-
HS37 (NA1,3C)	+	-	+	+	+
HS37 (NA1)	-	-	-	+	+
HS37[c]	-	-	-	+	-
165	+	-	+	-	-
164NT	-	-	-	-	-

[a] C_1, C_2, and D are C. botulinum type C and D toxins. Alpha and gamma are C. novyi type A toxins.
[b] Dominant toxin produced by C. botulinum type C strains.
[c] Cured of phage 3C.

The observation that phages from C. novyi type A could induce the production of C. novyi alpha toxin by a nontoxigenic derivative from C. botulinum type C suggested that the phages of C. novyi probably also govern toxigenicity of the C. novyi types. Subsequent studies at this laboratory confirmed the fact that the production of alpha toxin in C. novyi types A and B (types A and B are only C. novyi types producing alpha toxin) depended upon the constant participation of specific phages.

C. novyi is divided into 4 types designated as A to D. Type D closely resembles type B in respect to its toxin (beta, eta, theta), but it fails to produce alpha toxin. In a recent study, the phages from C. novyi type A were shown to infect type D strains, but the effect on alpha toxin production is not known.

Types C and D and C. novyi differ considerably from the other types of C. botulinum in the phage and host relationship. With these types C and D and the tox^+ bacteriophage/ bacterial relationship is pseudolysogenic, whereas the phages of the other C. botulinum types indicate that this relationship is true lysogeny.

Mechanisms Governing Toxigenicity of Proteolytic types A, A_F, B, and F and C. sporogenes

C. botulinum type A strain Boroff A has been cured of 4 different phages by treatment with mitomycin C-acridine orange or mitomycin-acriflavine. Additional phages cannot be induced and the bacteriophage-sensitive strain continues to produce toxin. Since type C and D strains produce more than one toxin and the bacteriophages only govern the production of dominant C_1 and D toxins, the possibility exists that more than one toxin might be produced by other types of C. botulinum. Antisera therefore was produced against the toxin(s) of the type A strain when it was cured of two phages. The antiserum, however, neutralized the undiluted toxin of the parent culture indicating that the same toxin(s) were produced by both the parent culture and the derivative strain which were cured of two phages. Research on the effect of antiserum against the toxin of the strain cured of 4 phages is planned.

We have purified each of these phages and currently have a host strain carrying each of the four phages. These phages will be used in later studies to determine the relationship of phages of proteolytic types A, B, A_F, F, and C. sporogenes. They will also be used in later experiments to test the sensitivity of cured strains of C. sporogenes.

A strain of type A cured of 4 different phages has been tested for sensitivity to phages of other type A strains and also to phages of type B, F, and C. sporogenes. The cured strain of type A was sensitive to phages of two different type A strains and one strain of type B, but was not sensitive to phages of type F and C. sporogenes.

The type A culture infected with these different phages was tested to determine the effect of the phages on toxin production. None of these phages affected the toxicity or type of toxin produced.

A strain of proteolytic type F (Langeland strain) cured of two phages has also been tested for its sensitivity to other phages. This strain appears to be sensitive to phages from four different strains of type A but not to type B or C. sporogenes. We do not know if these phages have any effect on the type of toxin produced. These results are, however, interesting because they show the close relationship of A, B, and F cultures and also because there is one strain of type A (isolated from Argentina) that produces small amounts of type F toxin. This culture is designated type A_f.

Lytic procedures have been developed for Clostridium botulinum types A, F, and C. sporogenes. Plasmids have been detected in all of the 5 strains of type A and one strain of proteolytic type F examined. A plasmid of similar size was also found in one of the five A-like cultures (resembling type A except for absence of toxigenicity), and in one of the nine C. sporogenes strains examined. The function of these plasmids will be studied in future experiments.

C. sporogenes and A-like organisms which resemble C. botulinum types A, B, and F except for the absence of the toxigenic characteristics have been tested for sensitivity to phages of 18 strains of type A, 14 strains of

type B, 3 strains of type F, and 1 strain of type A_F. None of these strains were sensitive to the phages of C. botulinum. It is entirely possible that some of the C. sporogenes strains may be carrying phages antigenically related to the C. botulinum phages and are therefore immune to infection.

Bacterial mutants defective in the production of protein toxins are very difficult to isolate, primarily because toxin production usually confers no known selective advantage for bacterial growth. Screening of cultures for toxigenic and nontoxigenic isolates could be greatly facilitated by assay methods performed directly on colonies grown in agar. High titer antisera prepared from purified toxin was recently obtained from Japan. This antisera was used to determine whether a precipitin reaction can be used to differentiate the toxigenic and nontoxigenic strains. Both toxigenic and nontoxigenic strains gave similar precipitin reactions. We therefore were unable to use the procedure for the isolation of nontoxigenic strains.

Mechanisms Governing Toxigenicity of Nonproteolytic Types B, E, and F

In order to determine the relationship of bacteriophage and toxicity, nonproteolytic strains of Clostridium botulinum types B, E, and F have been cultured in media containing mitomycin C and acridine orange and treated with ultraviolet light to cure the bacteria of their prophages. One strain of type B has been cured of one prophage but continues to carry a second prophage and to produce toxin. Another strain of nonproteolytic type B continues to carry its prophage after treatment with acridine orange, but produces toxin which requires treatment with trypsin before toxicity can be detected.

Nontoxigenic derivatives have been isolated from several other strains of type B, but these derivatives are not sensitive to the phages of the toxigenic parent culture. Numerous unsuccessful attempts have been made to convert these nontoxigenic derivatives to the toxigenic state. Our past experience with this group of microorganisms and the high frequency of isolating nontoxigenic derivatives from these two different toxigenic type B strains has suggested that plasmids or unidentified phages could be governing toxigenicity. These nontoxigenic derivatives have been induced to lyse with mitomycin C and tailless phage heads have been observed in electron micrographs. Phages have also been isolated from marine sediments that contain nonproteolytic type B spores; but none of the six different phages isolated would induce toxigenicity.

Nontoxigenic derivatives have also been isolated from a strain of type E. This isolate is not phage-sensitive and electron micrographs show that both the toxigenic and nontoxigenic strains carry the same phage.

The nontoxigenic derivatives and toxigenic parent cultures of both types B and E have been lysed to determine whether they harbor covalently closed circular DNA and whether there is a correlation between the presence of these plasmids and toxigenicity. Plasmids were observed in both the toxigenic and nontoxigenic strains. Several additional lytic and plasmid isolation procedures had to be used, however, to demonstrate plasmids in the lysates of the nontoxigenic strains.

In order to verify their nontoxigenicity, the type B strains were cultured in dialysis tubing bathed in broth, a system which increases the concentration of the toxin produced. Supernatant fluids from these cultures were tested for toxicity before and after being concentrated further by

dialysis against polyethylene glycol. Low titers, 5 to 20 MLD of toxin, were in several of these cultures after concentration by polyethylene glycol. These toxins, however, are different from the dominant toxins of types B and E and are not neutralized by type B or E antiserum. Furthermore, these toxins produce different symptoms in mice than the dominant toxins. It therefore appears that this may be a minor toxin not previously recognized and may be a similar phenomenon as observed in type C and D cultures.

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APPENDIX

ABSTRACTS OF PAPERS THAT ARE CURRENTLY BEING WRITTEN ON BACTERIOPHAGES AND TOXIGENICITY OF *C. BOTULINUM* TYPES C AND D AND *C. NOVYI* TYPES A AND B AND A LIST OF PUBLICATIONS IN PREPARATION.

ABSTRACTS OF PAPERS IN PREPARATION

Production of Different Toxins by Clostridium botulinum Types C and D Cultures and Their Nontoxigenic Derivatives Infected with Specific Bacteriophages

When C. botulinum type C and D cultures are cured of their bacteriophages, they simultaneously cease to produce the dominant C_1 and D toxins. These bacteriophage-sensitive strains continue to produce other minor toxins which were previously masked by the dominance of the C_1 and D toxins. These strains have also become sensitive to bacteriophages of other bacterial strains which induce the production of additional toxins. Based upon the production of different toxins and toxin combinations, eight subtypes of C. botulinum type C and eight subtypes of type D are now recognizable. Types C and D share three of these subtypes. Prior to these studies, only type D and two subtypes of type C were reported.

Production of Different Toxins by a Derivative of Clostridium botulinum Type C Infected with Different Bacteriophages

A derivative of Clostridium botulinum type C strain 162, designated as HS37, simultaneously ceased to produce C_1 and D toxins when it became sensitive to the parent bacteriophage. Strain HS37, however, continued to produce the gamma toxin which was neutralized by Clostridium novyi type A antiserum. Strain HS37 was sensitive to phages $3C^{tox+}$ from C. botulinum type C, to phage $1D^{tox+}$ from C. botulinum type D, and phage $NA1^{tox+}$ from C. novyi type A. When strain HS37 was infected with phage $NA1^{tox+}$, it became immune to the infection by the homologous phage, but continued to be sensitive to

phages 3C and 1D. Strain HS37 therefore could simultaneously carry phages NA1 and 1D or phages NA1 and 3C. By substitution of the phages, strain HS37 was induced to produce the following toxins. (Toxins are listed according to titer produced; C_1 and D toxins are C. botulinum type C and D toxins and gamma and alpha toxins are C. novyi toxins.) (1) HS37 produced gamma toxins; (2) HS37(3C) produced C_1, D, and gamma toxins; (3) HS37(1D) produced D, C_1, and gamma toxins; (4) HS37(3C,NA1) produced C_1, alpha, gamma, and D toxins; (5) HS37(1D,NA1) produced alpha, D, gamma, and C_1 toxins; and (6) HS37(NA1) produced alpha and gamma toxins. The toxigenicity and type of toxin produced by these cultures depended upon the continued presence of specific bacteriophages.

LIST OF PUBICATIONS IN PREPARATION

Eklund, M. W., F. T. Poysky, and M. E. Peterson. Production of different toxins by a derivative of Clostridium botulinum type C infected with different bacteriophage. In preparation for Infection and Immunity.

Eklund, M. W., F. T. Poysky, and M. E. Peterson. Production of different toxins by Clostridium botulinum type C and D cultures and their non-toxigenic derivatives infected with specific bacteriophages. In preparation for Infection and Immunity.

Eklund, M. W., and F. T. Poysky. Host range and characteristic of bacteriophages of Clostridium botulinum types C and D. To be written for Infection and Immunity.

Eklund, M. W., M. E. Peterson, and F. T. Poysky. Plasmids of Clostridium botulinum. To be written in 1979-1980 for Infection and Immunity.

Eklund, M. W., F. T. Poysky, and M. E. Peterson. Relationship of bacteriophages to toxigenicity of proteolytic Clostridium botulinum types A, B, and F. To be written in 1979-1980 for Infection and Immunity.